Supplemental Information to: Reversible, specific, active aggregates of endogenous proteins assemble upon heat stress, Wallace et al., *Cell*, 2015

S1 Extended Experimental Procedures

S1.1 Comparison to previous proteome-scale measurements of heat-induced protein aggregation

Our rapid kinetic measurements capture aggregation behavior distinct from that observed after longer stresses. As reported in the main text, our conditions capture aggregation in the majority of known heat-shock granule components forming at the same timescale. A recent study reported 117 proteins forming aggregates after two hours at 42°C (O'Connell et al., 2014); in these data, Pab1 is the only HSG component identified. We detect 90 of these 117 proteins, yet identify aggregation in only three (Pab1, Ura8, Tum1). These results indicate that aggregation measured at different times differs, or that the experimental protocols are incompatible. The two-hour measurements occur long after production of molecular chaperones, whose presence is expected to remodel, reverse, and prevent further aggregation of many proteins; by contrast, our measurements precede detectable induction of molecular chaperones.

S1.2 Yeast growth, heat shock, and cell fractionation

Yeast strain BY4741 was grown, with shaking, at 30°C in SC complete media in a baffled Erlenmeyer flask, to mid-exponential phase ($OD_{600} \approx 0.5$). 50 mL cell culture was transferred to a 50 mL conical tube and centrifuged for 1 min at $2,500 \times g$ at RT, and the media decanted. The tube containing cell pellet with residual medium was placed in a water bath at specified temperature (30°C, 37°C, 42°C, 46°C) for the specified amount of time (0, 2, 4, 8 minutes), after which the pellet was resuspended in 1 mL ice-cold Buffer S0 (120 mM KCl, 2 mM EDTA, 20 mM HEPES-KOH, pH 7.4), transferred to a chilled 1.5 mL microcentrifuge tube, and centrifuged again for 30 seconds at $5,000 \times g$, 4°C. The supernatant was discarded and pellet was resuspended in 100 μL Buffer S [Buffer S0 + 0.5mM DTT, 1:100 protease inhibitor cocktail IV (Calbiochem 539136), 1mM PMSF], divided in half and flash-frozen into two half-aliquots. One aliquot here became the total protein sample (T), resuspended in 400 μL Buffer T [20mM HEPES-NaOH, pH7.4, 150 mM NaCl, 3% SDS, 5 mM EDTA, 2 mM DTT, 1:100 PMSF, 1:1000 protease inhibitors IV), and lysed by boiling for 20 minutes at 95°C and vortexing. The other aliquot was placed in a 2 mL Eppendorf "Safe-Lok" tube containing a 7 mM stainless steel ball (Retsch) pre-chilled in liquid nitrogen (LN). Cells were lysed with $4 \times 90s \times 30Hz$ pulses in a Retsch MM100 mixer mill, chilling in LN between pulses. 400 μL ice-cold Buffer S was added, then the thawed lysate was clarified by centrifugation at $3,000 \times g$ for 30 seconds at 4°C. The clarified supernatant was transferred to a 1.5mL ultracentrifuge tube, and centrifuged at $100,000 \times g$ for 20 minutes at 4°C; the aqueous portion of this is the supernatant (S) sample. The pellet was washed in 500 μL Buffer S, and centrifuged again at $100,000 \times g$ for 20min at 4°C. The remaining pellet was mixed with 500 μL Buffer P [8 M urea, 20 mM HEPES-NaOH, pH7.4, 150 mM NaCl, 2% SDS, 2 mM EDTA, 2 mM DTT, 1:100 PMSF, 1:1000 protease inhibitors IV), by vortexing vigorously for 30 minutes. The resuspended pellet was centrifuged at $20,000 \times g$ at RT for 5 minutes, and the aqueous phase was designated as the pellet (P) fraction. Protein from total, supernatant, and pellet fractions was precipitated by chloroform/methanol extraction (Wessel and

Flügge, 1984).

SILAC recovery assay

Yeast auxotrophic for arginine and lysine (RK) were grown with light (rep. 2, heavy) isotope labeled RK (Cambridge Isotope Laboratories) at 30°C to mid-exponential phase, transferred to heavy (rep 2, light) isotope labeled RK, heat shocked for 10 minutes at 42°C, and allowed to recover at 30°C for 0, 20, 60 or 180 minutes. For the 180 minute timepoint in rep 2, cells were diluted in pre-warmed labeled media to ensure they stayed in exponential phase. Cells were harvested, mixed evenly with unheated cells grown in medium-isotope-labeled RK, and flash-frozen. Mixed samples were lysed and fractionated as above, and only the supernatant fraction was chloroform-methanol extracted, trypsin digested, and submitted for LC-MS/MS.

S1.3 Sample preparation for mass spectrometric analysis

Samples were measured to 100 μg of total protein each. Samples were digested with trypsin using a FASP protocol (Wiśniewski et al., 2009). For dimethyl labeling of T, S, P, samples, digested samples were labeled as described by Boersema et al. (2009); total was labeled +28Da (light), supernatant was labeled +32Da (medium), and pellet was labeled +36Da (heavy). Subsequently, T, S, P, samples from the same experiment were mixed evenly.

An aliquot of each sample was taken, and submitted directly for mass spectrometry analysis. The remaining sample was fractionated by high-performance liquid chromatography (HPLC). The HPLC 1200 Agilent system with fraction collector (Agilent Technologies, Santa Clara, CA) was used for ERLIC (electrostatic repulsion-hydrophilic interaction chromatography, Alpert (2008)) separation on a PolyWAX LP column (200 x 2.1 mm, 5m, 300, PolyLC Inc, Columbia, MD). Sample was fractionated into 20 fractions on a 70 minute LC gradient. Individual or combined fractions were submitted for mass spectrometry analysis.

S1.4 Mass spectrometry

Mass spectra were measured on an Orbitrap Velos Pro (Thermo Fisher, San Jose, CA). Samples injected from an autosampler (Waters, NanoAquity, Milford, MA) were loaded into the trapping column (75 μm column ID, 5 cm packed with 5 μm beads on 200 pores, from Michrom Bioresources, Inc.), washed for 15 minutes and then eluted to an analytical column with a gradient from 2 to 32% of buffer B [0.1% formic acid in acetonitrile] over a 90 minute gradient for each fraction. Instrument was set up to run TOP 20 method for MS/MS in the ion trap with an exclusion function turned on, followed by a MS1 scan in Orbitrap with 60K resolving power at mass of 400 m/z.

Obtained runs were analyzed jointly by MaxQuant Software, version 1.5.0.30 (Cox et al. (2011), http://maxquant.org/). Searches were done against verified and uncharacterized ORFs from the R64-1-1 release of the S288C genome proteome database (yeastgenome.org), and common contaminants added to the database from the Global Proteome Machine (http://www.thegpm.org/crap/). Searches were done with trypsin enzyme specificity, allowing 2 missed cleavages. Possible modifications included in the search parameters were: protein N-terminus acetylation, methionine oxidation, deamidation of asparagine and glutamine amino acids, and phosphorylation of serine, threonine, and tyrosine. For triplex dimethyl labeling, labels on primary amines for light (+28 Da), medium (+32 Da) and heavy (+36 Da) were searched for as variable modifications. For SILAC, labels of medium lysine (+4 Da), heavy lysine (+8 Da), medium arginine (+6 Da) and heavy arginine (+10

Da) were searched for as variable modifications. The database search criteria were held at 1% FDR on both protein and on peptide levels for all output reported data. All parameters for MaxQuant runs are supplied in the Dryad package.

We used MaxQuant intensities from the evidence.txt file, not the reported ratios which are calculated using a different method, because we noted a detection-intensity-dependent bias in ratios that was largely absent from the quotient of the intensities.

S1.5 Statistical analysis

MaxQuant reports intensities in three channels per detection event: light (L), medium (M), and heavy (H), which in this experimental design are noisy proxies for total sample (T), 100,000 × g supernatant (S), and 100,000 × g pellet (P) respectively. Our goal is to estimate the proportion in supernatant (pSup), the ratio of supernatant to total, for each protein individually, which is a number between 0 and 1. This is complicated by measurement noise in the data, principally that the separate samples undergo multiple processing steps in parallel before being mixed and measured by mass spectrometry, so that the ratios measured on the machine are not stoichiometric compared to the original ratios in cell lysate. For example, directly estimating the S/T ratio from the M/L ratio produces estimates greater than 1; conversely, we can employ conservation of mass (in cells, for each protein $T = S + P$) to constrain models in order to fit the data accurately.

We employed multiple statistical analyses to estimate pSup, finding that correcting for uneven fraction mixing, batch effects, and other measurement noise produces the most biologically coherent quantitative picture of protein aggregation across multiple biological replicates, while agreeing in outline with more naive analyses. Three alternative estimates of pSup are shown in figure S1. The first, called M/(M+H), simply takes the median across peptides of intensity ratios. The second, called model 2 (m.2), uses conservation of mass and an error model, applied to each experiment independently, to correct for uneven mixing and estimate confidence intervals. The third, called model 3 (m.3), corrects for batch effects in biochemical separation by normalizing the pSup across experiments for a subgroup of proteins. We found that all models agree in outline and produce high correlation (Pearson's r coefficient) between biological replicates, although model 2 successfully accounts for conservation of mass, and model 3 was better at reducing bias and root-mean-squared-error (RMSE), as shown in figure S1. In particular, model 3 reduced batch-effect variation between measurements of one biological sample with multiple treatments (30C.rep1, 46C.2min, 46C.4min, 46C.8min) and another collected at a different time and measured in less depth (30C.rep3, 37C.8min, 42C.8min). We use the output of model 3 as pSup estimates in all figures in this paper except as noted in fig. S1.

S1.5.1 Technical details of statistical models

The goal of the statistical analysis is to estimate proportion in supernatant (pSup), the ratio of supernatant to total. The measurement noise has three components, all acting multiplicatively. The first noise component captures the fact that the proportions of each sample (T, S, P) as mixed and measured differ from their original proportions in cell lysate, since samples are extracted, digested and labeled separately; we call these the mixing ratios, and denote them by $\vec{\alpha} = (\alpha_T, \alpha_S, \alpha_P)$. The second noise component quantifies how distinct peptide states from a single protein may have highly variable intensities; we call this the detectability of events, and denote by γ_{ij} for event j associated with protein i. The third, residual, noise component, denoted by $\epsilon_{C,ij}$, is considered independent across channels $C = (L, M, H)$, and for each detection event j associated with protein i.

The list of quantities needed for an accurate statistical model is as follows:

i indexes proteins
r_i proportion in supernatant (pSup) of protein i
T_i abundance of protein i
S_i abundance in supernatant of protein i
P_i abundance in pellet of protein i
j indexes peptide detection events
L_{ij} intensity in light channel detected in event j, protein i
M_{ij} intensity in medium channel detected in event j, protein i
H_{ij} intensity in heavy channel detected in event j, protein i
γ_{ij} detectability for event j
$\epsilon_{L,ij}$ residual noise in L intensity for event j
$\epsilon_{M,ij}$ residual noise in M intensity for event j
$\epsilon_{H,ij}$ residual noise in H intensity for event j
α_T mixing ratio for total fraction
α_S mixing ratio for supernatant fraction
α_P mixing ratio for pellet fraction

where $(\alpha_T, \alpha_S, \alpha_P) \in$ Simplex, $r_i \in [0, 1]$ for each i, and the remaining quantities are non-negative. We posit multiplicative lognormal noise in each channel. The full model is as follows.

$$L_{ij} = \alpha_T \, T_i \, \gamma_j \, \epsilon_{T,ij} \tag{1a}$$
$$M_{ij} = \alpha_S \, S_i \, \gamma_j \, \epsilon_{S,ij} \tag{1b}$$
$$H_{ij} = \alpha_P \, P_i \, \gamma_j \, \epsilon_{P,ij} \tag{1c}$$
$$S_i = r_i \, T_i \tag{1d}$$
$$P_i = (1 - r_i) \, T_i \tag{1e}$$

The inferential targets are the proportions in supernatant for each protein, $\{r_i\}$.

In fig. S1, model 1 "M/(M+H)" naively estimates $r_i = \text{median}_j \left(\frac{M_{ij}}{M_{ij} + H_{ij}} \right)$, which does not account for the mixing ratios $\vec{\alpha}$ nor use data from the total protein channel.

To estimate r_i, we need not estimate the absolute protein abundances. Thus we consider a restricted model for ratios $\frac{M}{L}$ and $\frac{H}{L}$ in our analysis, as follows.

$$\frac{M_{ij}}{L_{ij}} = \frac{\alpha_S}{\alpha_T} \, r_i \, \epsilon_{M/L,ij} \tag{2a}$$
$$\frac{H_{ij}}{L_{ij}} = \frac{\alpha_P}{\alpha_T} \, (1 - r_i) \, \epsilon_{H/L,ij} \tag{2b}$$

The restricted model has the advantage that both the absolute protein abundances, $\{T_i\}$, and the detectability parameters, $\{\gamma_{ij}\}$, cancel out, and need not be estimated. We complete the specifications by positing a single noise term per ratio, $\epsilon_{M/L,ij}$ and $\epsilon_{H/L,ij}$, for each event j associated with protein i. This strategy for estimating the proportions in supernatant is akin to a partial likelihood approach.

To complete the model specifications, we posit the following prior distributions:

$$r_i \overset{iid}{\sim} Beta(\tfrac{1}{2}, \tfrac{1}{2}) \tag{2c}$$

$$(\alpha_T, \alpha_S, \alpha_P) \sim Dirichlet(100, 100, 100) \tag{2d}$$

$$\begin{pmatrix} \ln \epsilon_{M:L,ij} \\ \ln \epsilon_{H:L,ij} \end{pmatrix} \overset{iid}{\sim} N\left(0, \begin{pmatrix} \eta_{M:L}^2 & \rho\eta_{M:L}\eta_{H:L} \\ \rho\eta_{M:L}\eta_{H:L} & \eta_{H:L}^2 \end{pmatrix}\right) \tag{2e}$$

$$\eta_{\bullet:L} \sim Cauchy(0, 1)^+ \tag{2f}$$

$$\rho \sim Beta(1, 1). \tag{2g}$$

Here we chose a $Beta(\frac{1}{2}, \frac{1}{2})$ prior for r_i, the Jeffreys prior for the binomial likelihood; after testing a variety of priors we found this to be weakly informative and numerically stable. The Dirichlet prior for $\vec{\alpha}$ is a strong prior that enforces even mixing proportions; since we have thousands of observations the posterior is nevertheless dominated by the data. We chose a half-Cauchy prior distribution for the variance parameters η as this is a sensible default choice for top-level variance parameters (Polson and Scott, 2012). ρ represents the correlation of the M:L and H:L ratios (equations 2a, 2b) due to shared noise from the L channel; if all noise variances are the same in each channel then $\rho = 0$.

We used equations (2a-2g) to estimate values of $\{r_i\}$ jointly with other specified parameters by Markov Chain Monte Carlo (Robert and Casella, 2005). We implemented the sampler using the probabilistic programming language STAN, accessed using the rstan package (Stan Development Team, 2014) in the R software environment (R Core Team, 2014). This output r_i is displayed as pSup, model 2 in fig. S1. All code is provided in the datadryad package.

We fit model 2 to each experiment individually. Subsequently, we found discrepancies in certain datasets consistent with compression of dynamic range (fig. S1), presumably due to less efficient separation, an artifact of the mass spectrometric measurement and MaxQuant analysis, or other batch effects. To correct for this, we chose lists of proteins reliably in the supernatant (≥ 2 peptides detected in every experiment, and $r_i > 0.9$ in 30C.rep1 and 46C.8min) or reliably in the pellet (≥ 2 peptides detected in every experiment, and $r_i < 0.1$ in 30C.rep1 and 46C.8min), with median r_i's F_S and F_P respectively across the 30C.rep1 and 46C.8min datasets. Then, we normalized so that these proteins had the same pSup across all experiments, with a linear transformation in log-odds space. Precisely, for a given experiment, f_S is the median r_i for reliably supernatant proteins and f_P for reliably pelleted proteins, and

$$g(r) = \log\left(\frac{r}{1-r}\right) \tag{3}$$

is the logistic function, we transformed:

$$r_i' = g^{-1}\left(g(F_P) + [g(r_i) - g(f_P)]\,\frac{g(F_S) - g(F_P)}{g(f_S) - g(f_P)}\right). \tag{4}$$

This output r_i' is displayed as pSup, model 3 in fig. S1 and reported in the data package, along with 95% confidence intervals. As the figure shows, it is a minor adjustment in most datasets, and generally reduces the inter-dataset bias and RMSE without obscuring the signal of heat-dependent aggregation. 95% confidence intervals from model 2 were transformed by applying the same equation and parameters to the endpoints. Processing code/scripts and intermediate data are included in the data package.

S1.6 Statistical analysis for SILAC recovery data

In the SILAC recovery assay, we again used MaxQuant Software, version 1.5.0.30 (Cox et al., 2011). We searched for SILAC-labeled arginine and lysine as standard, otherwise using the same variable protein modifications as above. We report median ratios of MaxQuant-estimated intensities, correcting for deviations from even mixing by fixing the median ratio to 1 for proteins reliably found in the supernatant in the aggregation assay previously. This code is also available in the data package.

S1.7 Sedimentation coefficients of pelleting particles

Here we estimate the particle sizes expected to sediment in our assay. Centrifugation conditions are acceleration $a = 100,000g \approx 10^6 ms^{-2}$ for $t = 20\text{mins} \approx 10^3 s$.

0.5mL of liquid in a 1.5mL eppendorf tube is approximately $d = 2cm = 2 \times 10^{-3} m$ high, so particles pellet if:

$$v = d/t \geq \frac{2 \times 10^{-3} m}{10^3 s} = 2 \times 10^{-6} ms^{-1} \tag{5}$$

since the sedimentation coefficient $c = v_t/a$, that implies that

$$c \geq \frac{2 \times 10^{-6} ms^{-1}}{10^6 ms^{-2}} = 5 * 10^{-11} s = 500S \tag{6}$$

as a Svedberg unit $S = 10^{-13} s$.

This rough estimate suggests that the smallest pelleting particles should be much larger than 80S ribosomes, consistent with our observations.

S1.8 Protein annotation

Annotation of protein groups used in figures 1 and S4, were derived from the Saccharomyces Genome Database (Cherry et al., 2012) for most groups, from the sources listed in table S2 for heat shock granule components, and from computational structure prediction for Membrane proteins.

Glycolytic enzymes are: Hxk1, Hxk2, Pgi1, Pfk1, Pfk2, Fba1, Tpi1, Tdh3, Tdh2, Tdh1, Pgk1, Gpm1, Eno1, Eno2, Cdc19, Pyk2.

Ribosomal proteins annotated here include only core components, whose names in yeast begin Rpl for the large subunit, Rps for the small subunit, and Rpp for the stalk.

Our list of nucleolar proteins is manually curated from the gene ontology category (Cherry et al., 2012), as proteins whose *principal* function is nucleolar. There are 143 well-detected nucleolar proteins: Mak16, Utp20, Mak5, Enp1, Spb1, Krr1, Bud23, Pwp2, Rsa4, Csm1, Ycr087c-a, Nop1, Dbp10, Tsr1, Nop14, Sas10, Nhp2, Nop6, Fal1, Mak21, Rrp8, Arx1, Fob1, Rpa14, Hmo1, Bfr2, Ssf2, Utp4, Fcf1, Esf1, Utp5, Utp6, Snu13, Pol5, Nop16, Nug1, Utp7, Spb4, Loc1, Cdc14, Dbp3, Prp43, Rok1, Utp22, Nop7, Utp8, Enp2, Mtr3, Nsr1, Nop19, Efg1, Pxr1, Ygr283c, Rrp3, Ssf1, Nop10, Rpf1, Gar1, Rpc10, Imp3, Dbp8, Utp9, Air1, Utp25, Rrt14, Nop9, Hca4, Mtr4, Utp18, Net1, Utp10, Rpa34, Mpp10, Urb2, Rpa12, Mrt4, Urb1, Dhr2, Rrp14, Utp11, Rrn3, Ebp2, Tof2, Dbp7, Las1, Rpf2, Srp40, Drs1, Sof1, Rix7, Noc3, Rlp24, Fcf2, Dip2, Acs2, Cbf5, Emg1, Pwp1, Nop56, Rsa3, Utp13, Ifh1, Dbp9, Utp21, Fpr4, Fpr3, Utp14, Utp15, Ecm16, Rrb1, Rrp5, Tma23, Has1, Rlp7, Imp4, Nop15, Rpc19, Kre33, Nop13, Ubp10, Rpa49, Trf5, Kri1, Dbp6, Nog2, Esf2, Rcl1, Nop12, Brx1, Pap2, Nop8, Utp23, Bud21, Pno1, Ytm1, Rrs1, Nop58, Rpa43, Rpa190, Nop4, Nog1, Nan1, Nop53, Nip7, Bms1, Dim1, Rpa135, Tif6, Mrd1, Rrp9, Rrp15, Noc4.

Our list of membrane proteins includes those with at least 2 transmembrane domains identified by TMHMM 2.0 (Krogh et al., 2001). There are 268 well-detected membrane proteins: Aus1, Ccc1, Drs2, Rcf2, Erg11, Sna2, Yro2, Gup1, Ftr1, Eos1, Gaa1, Rsn1, Tda5, Csg2, Sft2, Tvp38, Chs2, Alg9, Adp1, Nce102, Gpi11, Erp2, Sal1, Spf1, Dip5, Nsg1, Pmt7, Ecm3, Dfm1, Ssh1, Pmt3, Dnf1, Cst26, Sly41, Izh2, Erg3, Ale1, Pam17, Yip3, Gpi14, Tul1, Mal11, Neo1, Mdl1, Alg12, Dpp1, Ste24, Pdr15, Vma3, Erp5, Fat1, Tcb1, Hip1, Rtn1, Tsc13, Avt3, Ymd8, Aim26, Ost2, Ste2, Qdr2, Fre1, Vtc2, Vcx1, Lnp1, Shy1, Atg33, Kha1, Lac1, Sec61, Fth1, Dfg10, Atm1, Nnf2, Zrt2, Yor1, Zrt1, Flc1, Sam3, Cds1, Nte1, Pmc1, Ncr1, Gpt2, Pma1, Vtc3, Lem3, Ndc1, Cpt1, Fks1, Brl1, Hmg2, Lyp1, Atr1, Mup1, Cox15, Usa1, Tat1, Ena1, Sec62, Ypk9, Sey1, Tna1, Ost5, Aur1, Itr1, Ost3, Ssm4, Gdt1, Bap2, Zrc1, Ptr2, Sac1, Pmr1, Pom152, Hmg1, Pdr5, Fre7, Alr2, Pmt1, Enb1, Agp1, Emc4, Cho2, Sur2, Fks3, Emp24, Chs1, Ybt1, Gpi17, Yos1, Erv29, Ecm33, Hxt5, Dnf3, Gex2, Chs3, Tvp18, Svp26, Vma9, Cwh43, Pma2, Cos10, Alg2, Scs7, Gtt3, Rer1, Aac1, Cdc50, Stt3, Bi4, Ctr1, Spo75, Die2, Alg3, Pom33, Pmt5, Arn2, Pmt6, Tpo1, Hxt1, Cox1, Cox10, Erg4, Vph2, Rcf1, Pdr12, Vtc4, Bpt1, Sur4, Get2, Uip3, Mal31, Cpr8, Pho87, Rax1, Sec63, Hxt16, Mrl1, Swp1, Vmr1, Sdh3, Erg28, Pis1, Fmp37, Nsg2, Erg1, Avt7, Fsf1, Lag1, Sct1, Fun26, Ato3, Avt1, Bap3, Mrh1, Yip4, Gpi1, Erv41, Thi72, Lcb3, Pho91, Erp1, Cox2, Crd1, Trk1, Akr1, Ptm1, Mnr2, Hxt6, Gsc2, Pmt2, Emc1, Ist2, Ycf1, Pet9, Vph1, Yip5, Yif1, Smf3, Emp70, Fen1, Fcy21, Ost6, Flc2, Yet1, Tpo4, Aim14, Pga3, Erj5, Ypq1, Ole1, Erd2, Rbd2, Aac3, Stv1, Pex31, Yct1, Sur7, Vba4, Mcd4, Dnf2, Gup2, Tpo3, Gab1, Pmt4, Syg1, Ste6, Hxt10, Tmn3, Mtc7, Gnp1, Spc1, Yop1, Rim21, Snq2, Cdc1, Cho1, Yet3, Elo1, Erv14, Mdl2, Hxt3, Flc3, Pho86, Msc2, Spc2, Dal4.

Our list of molecular chaperones is: Ssa1, Ssa2, Ssa3, Ssa4, Ssb1, Ssb2, Sse1, Ssz1, Hsp26, Hsp42, Hsp82, Hsc82, Hsp104, Zuo1, Sse2, Fes1, Ydj1, Sis1, Hsp78, Ssc1, Kar2, Sil1, Hch1, Aha1, Sba1, Sti1.

S1.9 Strains

To construct fluorescently tagged strains for microscopy, plasmids pJLS033 and pJLS035 were constructed for C-terminal Clover and mRuby2 labeling at the native locus. Clover/mRuby2_KanMX cassette PCR fragments were transformed into BY4741 and BY4742 according to standard lithium acetate protocol and selected using G418. Diploids were generated by crossing PCR- confirmed positives for 4 hours at RT, then overnight at 30°C on YPD, followed by selection on SC –cys –met –lys plates. All strains used are listed in Table S6.

S1.10 Spinning-disk confocal fluorescence microscopy

Cells were grown to mid-log phase ($\approx 3 \times 10^7$ cells/mL) in non-fluorescent synthetic yeast growth medium (NSD; per 1 L: 20 g glucose, 5 g ammonium sulfate, 0.79 g CSM [Sunrise Science Products #1001-100], 1.7 g YNB trace elements [US Biological Y2035-01], 2 mL 500x non-fluorescent vitamin mix [500 mg calcium pantothenate, 2.5 g myo-inositol, 100 mg niacin, 50 mg p-aminobenzoic acid, 100 mg pyridoxine hydrochloride, 100 mg thiamine hydrochloride, dH_2O to 500 mL, filter sterilized], 2 mL 500x biotin (0.2 g/L), 2 mL 500× $CoCl_2$-$6H_2O$ (0.1 g/L), 20 mg adenine sulfate). 25 μL aliquots were heat-treated for 8 minutes at 30°C or 46°C in an Eppendorf Thermomixer. To reduce live cell motion while imaging, coverslips coated with concanavalin-A were applied to base-washed slides to prepare flow-chambers using melted Parafilm (Joglekar et al., 2008). Heat-treated cells were applied to the flow-chamber and allowed to settle before rinsing unbound cells with NSD mounting media and sealing with VALAP (equal parts Vaseline, lanolin, and paraffin wax mixed to homogeneity by gentle heating and applied using a cotton-tipped applicator) to decrease evaporation

of mounting medium.

Images were captured with a 100×/1.45 oil objective on Olympus DSU spinning disk confocal microscope (Olympus Corporation of the Americas, Center Valley, PA) with a Hamamatsu model C9100 EM-CCD camera (Hamamatsu Photonics, Skokie, IL) controlled by SlideBook v5.0 software (Intelligent Imaging Innovations, Denver, CO). Filter sets were FITC/Cy2 (excitation 490/20 nm, emission 528/38 nm) for Clover and DsRed (excitation 565/25 nm, emission 624/40 nm) for mRuby2. 20 plane z-stacks were collected over a range of 4.94 μm (step size 0.26 μm). Fluorescence images were deconvolved in Fiji software (Schindelin et al., 2012) using the deconvolution lab plugin (Vonesch and Unser, 2008) to perform 10 iterations of the Richardson-Lucy algorithm, subtracting minimal intensity background and using point-spread functions generated for the Olympus DSU microscope by PSF generator software (Kirshner et al., 2013). Then, using Fiji, a single slice from the deconvolved stack was selected, a $20\mu m \times 20\mu m$ square selected, and intensity automatically adjusted (ImageJ macro provided upon request); the corresponding single-slice square from the DIC images was selected alongside.

S1.11 Protein gel electrophoresis

Samples were first boiled in Laemmli buffer (BioRad #161-0737), and aliquots (5μL unless otherwise noted) were loaded onto 4-15 % Criterion TGX (BioRad #567-1084). Gels were run at 200V for 40 minutes in a Bio-Rad Criterion system. Coomassie staining was performed using Gelcode Blue (Thermo #24592) according to manufacturer's instructions. Gels were imaged using a Chemidoc-MP (Bio-Rad).

S1.12 Western blotting

Proteins were transferred to 0.2μm nitrocellulose membranes (BioRad #9004-70-0) in Towbin buffer using the Criterion blotter system (Bio-Rad). Protein was detected using 2.5 μg anti-Pab1 antibody (EnCor; Gainesville, FL; #MCA-1G1) , or 5 μg anti-Ssz1 antibody (Hundley et al., 2002), along with the ONE-HOUR Western^{TM} Basic Kit (Mouse; GenScript #L00205) according to manufacturer's instructions, and imaged on a Chemidoc-MP (Bio-Rad).

S1.13 Purification of multisynthetase complex components

Unless otherwise stated, cells of *E. coli* strain BL21 (DE3) were grown in LB at 37°C.

Arc1

The full-length Arc1 gene from *S. cerevisiae* was cloned into the pET28a vector using standard cloning methodology, and subsequently transformed into BL21 cells for expression as a fusion with an N-terminal 6-His tag. A single colony was used to inoculate 50 mL LB supplemented with kanamycin (50 μg/mL), and culture was grown to mid-log phase at 37°C prior to 2% inoculation of 1 L fresh LB + kanamycin. IPTG was added to a final concentration of 1 mM when the culture reached $OD_{600} = 0.75$, at which time the flask was transferred to 30°C incubator with shaking at 200 RPM for 4 hours. Cells were pelleted at $5000 \times g$ for 10 minutes at 4°C, then resuspended in 20 mM HEPES (pH 7.4), 120 mM KCl, 5 mM imidazole, 0.2% Triton X-100, 0.5 mM β-mercaptoethanol, and EDTA-free complete protease inhibitor tablets (Roche 05 056 489 001), then lysed on ice/water bath with sonication 7 seconds ON/7 seconds OFF cycles for 20 minutes at 60% amplitude. Cell debris and insoluble material was removed via centrifugation for 20 minutes at $18,000 \times g$, 4°C.

Clarified lysate was loaded onto a buffer-equilibrated 5 mL HiTrap Chelating HP column (GE Healthcare Life Sciences 17-0409) on an ÄKTAprime system (GE Healthcare Life Sciences) with automated fraction collector. The column was washed with 5 column volumes (CV) of buffer containing 20 mM HEPES, 120 mM KCl, 30 mM imidazole, then bound proteins were eluted over a 40 mL gradient (0-100%) to buffer containing 20 mM HEPES, 120 mM KCl, 300 mM imidazole. Fractions containing Arc1 were pooled and buffer exchanged to 50 mM Na_2HPO_4, pH 6.7, prior to loading onto a 5 mL HiTrap SP HP column (GE Healthcare Life Sciences 17-1151-01). The column was washed with 5 CV of buffer containing 50 mM Na_2HPO_4, pH 6.7, and Arc1 was eluted over a 35 mL gradient (0-100%) to buffer containing 50 mM Na_2HPO_4, 1M NaCl, pH 6.7. Fractions containing Arc1 were pooled, buffer exchanged to buffer A [20 mM HEPES, 150 mM KCl, , pH 7.4], concentrated and further purified on Superose 6 10/300 GL column (GE Healthcare Life Sciences) equilibrated with buffer A using ÄKTApurifier FPLC system (FPLC; GE Life Sciences). Arc1 was eluted at 15.8 ml and 0.5 ml peak fraction was divided into aliquots, frozen, and stored at −80°C until used.

Mes1

The protocol is the same as for Arc1, with the following exceptions. Culture was grown in TB medium [1.2% peptone, 2.4% yeast extract, 0.4% glycerol, 72 mM K_2HPO_4, 17 mM KH_2PO_4] and induced at OD_{600} = 0.4 with 1 mM IPTG at 20°C for 5 hours. Cells were lysed in buffer containing 20 mM HEPES, 250 mM KCl, 20 mM imidazole, 0.5 mM β-mercaptoethanol, 0.5% Chaps detergent and EDTA-free complete protease inhibitor tablets, pH 7.4]. HiTrap Chelating HP column was washed with buffer containing 20 mM HEPES, 250 mM KCl, 20 mM imidazole, pH 7.4 and bound protein was eluted using buffer containing 20 mM HEPES, 250 mM KCl, and 400 mM imidazole, pH 7.4]. Fractions containing Mes1 were pooled, buffer exchanged to 20 mM HEPES (pH 7.4), 80 mM KCl, applied to monoQ 5/50 GL column and fractions were collected across a 10 ml 80-600 mM KCl gradient by FPLC. Mes1 containing fraction (eluted between 300 and 400 mM KCl) was then applied to Superdex 200 10/300 GL equilibrated with buffer A and 0.5 ml fractions were collected following elution by FPLC. Mes1 was eluted at 14.0 ml and peak fractions were concentrated, divided into aliquots, frozen, and stored at −80°C until used.

Gus1

The protocol is the same as for Arc1, with the following exceptions. Cells were induced at OD_{600} = 0.75 with 1 mM IPTG at 20°C for 5 hours. Cells were lysed in buffer containing 20 mM HEPES, 140 mM KCl, 20 mM imidazole, 0.5 mM β-mercaptoethanol, 0.2% Triton X-100 and EDTA-free complete protease inhibitor tablets, pH 7.4]. HiTrap Chelating HP column was washed with buffer containing 20 mM HEPES, 140 mM KCl, and 20 mM imidazole, pH 7.4 and bound protein was eluted using buffer containing 20 mM HEPES, 140 mM KCl, and 400 mM imidazole, pH 7.4]. Fractions containing Gus1 were pooled and buffer exchanged to 20 mM bis-tris propane, pH 7.1, then loaded onto a 5 mL HiTrap Q HP column (GE Healthcare Life Sciences 17-1154-01). Column was washed with buffer containing 20 mM bis-tris propane, pH 7.1, and eluted using 20 mM bis-tris propane, 1 M NaCl, pH 7.1. Fractions containing Gus1 were pooled, buffer exchanged to buffer A, concentrated and further purified on Superose 6 10/300 GL column using FPLC. Gus1 was eluted at 16.2 ml and 0.5 mL peak fraction was divided into aliquots, frozen, and stored at −80°C until used.

Isolation and heat-assembly of AME multisynthetase complex

Purified Mes1, Arc1, Gus1 were mixed in a 1:1:1 molar ratio in a total volume of 0.5 mL in a buffer containing 20 mM HEPES (pH 7.4), 150 mM KCl, 5 mM $MgCl_2$, incubated for 12 hours at 4°C, then spun down at $10,000 \times g$ for 10 minutes at 4°C prior to loading on a Superdex200 column equilibrated with buffer B (20 mM HEPES [pH 7.0], 150 mM KCl, 0.1 mM $MgCl_2$). AME complex was collected upon elution from the column at 11.3 mL and subsequently concentrated and stored at 4°C.

All reactions were assembled to contain 4 μM AME (or 2μM Mes1) in buffer 20 mM HEPES (pH=7.0), 150 mM KCl, 2 mM $MgCl_2$ and incubated 15 min at either 30°C or 46°C followed by dilution (when indicated) and further 1 hr incubation at indicated temperature. Reactions were centrifuged at 100,000 g for 20 min and supernatant transferred to a clean tube. The pellets were washed once with 200 μL of 20 mM HEPES (pH=7.0), 150 mM KCl, 2 mM $MgCl_2$ and centrifuged again. Pellets were resolubilized in Laemmli buffer and proportional amounts of T and S material diluted with 2× Laemmli were subjected to PAGE and staining. Heated samples wer diluted 40× with 20 mM HEPES (pH=7.0), 150 mM KCl, 2 mM $MgCl_2$ followed by 1 hr incubation at 30°C, and in a separate sample additionally supplemented with (cold) methionine and yeast total tRNA as in aminoacylation reactions followed by 1 hr incubation at 30°C.

Dynamic light scattering

Dynamic light scattering (DLS) measurements were performed using a DynaPro Nanostar (Wyatt Technology). Protein samples at 5–6 μM in aggregation buffer were incubated at 20°C and centrifuged at 21,000 g for 30min prior to the measurements, equilibrated and verified for stability in DLS at 25°C, and then the temperature was ramped to 50°C at 0.25°C per minute. Each timepoint was measured five times, with an acquisition time of 6s, filtered for only those runs with a baseline deviation of less than 0.003 to remove spurious readings. The apparent hydration radii reported are cumulant radii calculated using Dynamics software (Wyatt Technology).

Absorbance

For aggregation studies, absorbance data was collected on a Jasco J-715 spectropolarimeter equipped with Jasco PFD-425S temperature control unit. Proteins were dialyzed into assay buffer (20mM HEPES, 175mM KCl, pH 7.4) and this buffer alone was preheated to the desired temperature in a 1cm quartz cuvette, with a magnetic stir bar at full speed to prevent settling of large particles during the experiment. Protein sample at the desired concentration was added after temperature equilibration, and 550nm absorbance readings were collected at 1-second intervals. Absorbance differences were calculated by subtracting the minimum of the first five readings.

Supernatant/pellet fractionation of AME

Aminoacylation assay

Filter-based aminoacylation reactions were performed at 30°C in 20mM HEPES-KOH, 150mM NH4Cl, 100uM cold Met, 10mM MgCl2, 0.1mM EDTA, 5mM DTT, 4mM ATP, 0.5 $\mu Ci/\mu L$ ^{35}S-methionine, 40 μM total yeast tRNA and AME or Mes1 enzyme. Heat shock was performed on enzymes at 4μM at 46°C for 15 minutes or 95°C for 5 minutes immediately prior to the aminoacylation reaction, then diluted to 10× the reaction concentration. Reactions were run for 10 minutes

and quenched in cold 10 % trichloroacetic acid (TCA) before spotting on filter disks in a vacuum apparatus. Filters were washed with 2 mL cold 10 % TCA and 1 mL cold ethanol, dried, and exposed to a phosphorimager screen for quantification with ImageLab software (BioRad).

Aminoacylation reactions for tRNA microarray analysis were performed at 30°C in 50 mM HEPES KOH (pH 7.5), 100 mM potassium glutamate, 10 mM magnesium acetate, 1 mM DTT, 2 mM ATP, 2.5 $\mu Ci/\mu L$ ^{35}S-methionine, 40 μM total yeast tRNA and 1 μM AME enzyme prepared as above. Microarray analysis of tRNA charging was performed as previously described (Netzer et al., 2009; Wiltrout et al., 2012).

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